EXHIBIT B

Functional protein microarrays

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Microarrays of immobilized functional proteins have the potential to increase dramatically the throughput of proteomic analysis. Micro-immunoassays, in which biological samples are exposed to arrays of immobilized antibodies, can be used for protein expression profiling. In addition, protein function can be elucidated by performing binding and enzymatic assays on arrays of biologically active proteins.

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Abbreviations
CD cluster of differentiation
ELISA enzyme-linked immunosorbent assay

Introduction

Biologists, chemists, materials scientists and engineers have collaborated to develop DNA microarrays, which make it possible, in theory, to monitor the expression levels of all genes in an organism simultaneously [1]. The key to this new technology has been the development of surface-based assays in which numerous probes are immobilized in a spatially addressable manner. Such assay formats lend themselves well to miniaturization and multiplexing. Similar approaches are now being employed for highly parallel, miniaturized protein analysis. In these cases, the immobilized material is protein or some substance capable of capturing proteins out of complex biological mixtures. The main benefits of these protein microarrays will be the amount of information that can be obtained as a function of research time and sample quantity. A single feature on a protein microarray may require the deposition of less than one picogram of protein, which is about one million times less than that typically used to coat one well of a 96-well microtiter plate. The theoretical benefits of protein microarrays and the history of their development are discussed in the review by Ekins in this issue. We limit ourselves here to applications in which proteins are arrayed onto surfaces in such a way that they maintain their native structures and activities. Other reviews in this issue cover arrays of peptides (see review by Frank) and tissues and cells (see review by Kallioniemi). For recent in depth reviews of protein microarrays, see [2,3•,4].

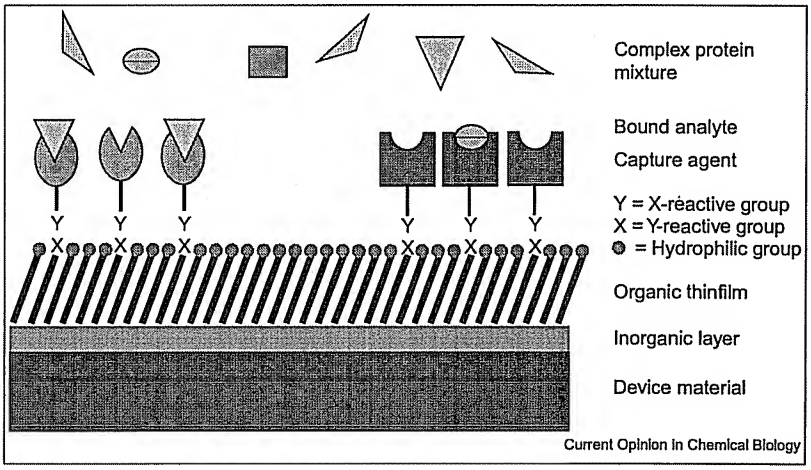
Although the invention of DNA arrays can provide inspiration and some technical leads for the development of protein biochips, the detailed methodology for building protein arrays will differ significantly from those made with DNA. The main reason for this is the tendency of proteins to undergo denaturation and to exhibit non-specific binding to surfaces. For these reasons, it is important to array proteins, if possible, without allowing them to denature because of dehydration or to contact with commonly used device materials such as steal, plastics, glass, etc. Miniaturization makes these issues far more important because the surface to volume ratio increases dramatically as one decreases the sample volumes to nanoliter and picoliter scales. Despite these difficulties, important advances have appeared in the past year, enabling impressive applications of this burgeoning technology.

Engineering surfaces for protein immobilization

The types of surfaces to which proteins can be immobilized fall into two categories. The first and simplest type of immobilization is onto surfaces that have a high inherent binding energy to proteins in general. The most common of these substrates are hydrophobic plastics such as polystyrene, to which most proteins physically adsorb by van der Waals, hydrophobic and hydrogen-bonding interactions. This type of physical adsorption is generally used to immobilize 'capture antibodies' in enzyme-linked immunosorbent assays (ELISAs). The advantage of this type of immobilization is that it is very simple to perform, because it does not require any modification of the protein for its attachment to the surface. The disadvantage is that most of the immobilized protein can be inactivated because of denaturation and steric occlusion [5,6]. The physical adsorption of proteins onto surfaces also tends to be heterogeneous, with proteins clustering together in patches [7]. Similar heterogeneity is observed on other surfaces used for non-covalent protein adsorption, such as nitrocellulose and poly-lysine-coated glass.

Physical adsorption of proteins onto surfaces results from a large number of weak contacts, and can influence protein structure and activity. A preferred method relies on one or a small number of strong bonds between the protein and surface, leaving the protein largely unaltered, except in the vicinity of the contact point. Examples include covalent attachment of proteins [8•], immobilization of biotinylated proteins onto streptavidin-coated surfaces [9••,10], and immobilization of His-tagged proteins (proteins containing a poly-histidine sequence) onto Ni^{2+}-chelating surfaces [11••]. One advantage of covalent and streptavidin–biotin-based attachment is that, following protein immobilization, the surface reactivity can be quenched by incubation with a small molecule. This can render the surface more protein-resistant than those with intrinsic protein-binding activity. Recent examples of covalent modification have utilized amine-reactive surfaces, which modify lysine sidechains [8•,12,13], and aldehyde-reactive surfaces, which react with oxidized glycoproteins [13].

Figure 1



Scheme for an idealized protein array. The underlying device material could be glass, silicon, or some other flat surface. A thin inorganic layer such as a noble metal or metal oxide is applied to this surface and can facilitate detection of protein-binding events (e.g. a gold layer for surface plasmon resonance). On top of this inorganic layer is assembled an organic thinfilm in which the omega functionality is hydrophilic [14]. This monolayer coats the chip surface, making it protein-resistant. The chip is patterned [41,42] in such a way, however, that it contains features that are coated by mixed monolayers in which a fraction of the omega functionalities possess a group (X) that can react with a moiety (Y) engineered into a specific site on the proteins to be immobilized. The engineered protein is therefore oriented on the surface such that the active site or binding site faces away from the thinfilm and can therefore interact optimally with proteins in the biological sample. The figure shows two features, each derivatized with a different capture agent, and separated by a completely protein-resistant patch. After immobilizing different proteins onto different patches, a complex protein mixtures can be applied to the chip and each feature will capture specific proteins while repelling others.

Surfaces can be engineered to avoid non-specific protein adsorption [14], while making specific interactions with proteins of interest. An optimal composition of a protein microarray based on these principles is outlined in Figure 1. One method for accomplishing this is to derivatize surfaces with poly(ethylene glycol), which is known to repel non-specific protein adsorption [14,15]. By derivatizing a fraction of the poly(ethylene glycol) chains with biotin, streptavidin can be specifically attached to the surface. Because streptavidin is tetrameric, biotinylated proteins can in turn be immobilized. This method of attaching specific proteins to otherwise protein-resistant surfaces has been used to generate high-density protein microarrays of very high quality [9••,10].

To increase the number of proteins that can be immobilized per unit area, Mirzabekov and colleagues attach gel pads to surfaces, thus giving the features a third dimension, preventing rapid evaporation and creating barriers between features. This approach is described by Mirzabekov in this issue.

Engineering proteins for surface attachment

The most common method for immobilizing proteins through covalent (or biotin-based) interactions is by randomly conjugating lysine residues on proteins to amine-reactive surfaces (or biotinylation reagents). When the structure of a protein is known, it may be advantageous to immobilize it in such a way that the protein is attached to the surface in a unique orientation in which the biologically important site faces away from the surface. This is most easily achieved when using recombinant proteins, in which amino- or carboxy-terminal tags can be introduced. A His-tag can be used to immobilize proteins in an oriented fashion [11••], as can an amino-terminal serine or threonine residue [16]. For non-recombinant proteins, one must take advantage of existing functional groups on the protein. Several methods have been developed to orient antibodies and their fragments on surfaces (reviewed in [17]). Antibodies contain a conserved glycosylation site on the Fc domain (the crystallizable domain of immunoglobulin G), and this can be oxidized and then biotinylated using biotin hydrazide. Alternatively, antibodies can be cleaved by pepsin into dimeric Fab′ fragments (antigen-binding fragments of immunoglobulin G) [18], which are linked together by disulfide bonds in the hinge region. The disulfides can be reduced and then conjugated to a thiol-reactive surface or biotin–maleimide. Using either of these methods, the antibodies or Fab′ fragments are oriented in such a way that the antigen-binding sites face away from the surface. Several studies have shown that these oriented antibodies retain a higher and more homogeneous level of activity than randomly oriented ones (see [19•] and references therein). Full-length antibodies can also be oriented onto surfaces through binding to immobilized protein A or G [19•], but this interaction may not be stable enough for some applications.

Methods for printing proteins

Robotic printing of proteins onto surfaces has been performed using instruments designed for DNA spotting [11••,20,21,22••,23,24]. One drawback to this approach is the long printing times that, coupled with the small volumes that are spotted onto the surfaces, generally result in drying of the protein spots. To ameliorate this problem, MacBeath and Schreiber [8•] humidified the spotting chamber and also added glycerol to the protein solutions. Other printing methods include deposition by a hydrogel

Table 1

Applications of functional protein microarrays.

Application	No. proteins	Detection	Refs
Antibody arrays			
Protein profiling (proof of concept)	115	Fluorescent analyte	[20]
Measurement of protein abundances in blood	3	Sandwich ELISA/ fluorescence	[24]
Measurement of cytokine abundances	7, 24	Sandwich ELISA/ chemiluminescence	[22•,35]
Capture of leukocytes/ phenotyping leukemias	60	Light microscopy	[36•]
Antigen arrays			
Reverse immunoassay to measure autoimmune antibodies	18	ELISA/ chemiluminescence	[25]
Reverse immunoassays to measure allergies	5*	Rolling circle DNA synthesis fluorescent probe	[38]
Immobilized proteins from cDNA libraries			
Kinase substrate specificity	119	Radioassay	[12]
Identification of calmodulin- and phospholipid-binding proteins	5800	Fluorescent probe	[11•]

*In this case, each feature was a complex protein mixture from common allergens.

stamper inked with an aqueous protein solution [25], inkjet printing [26,27], electrospray through a dielectric grid mask [28,29], and direct application of protein solutions via microfluidic networks [30,31•]. With all of these methods, the challenge is to keep the proteins hydrated at all times and to minimize the denaturing of proteins by various device materials and by the liquid–air interphase. This is an area with considerable need for innovation and improvement.

Applications of protein arrays

Recent applications of protein biochips are listed in Table 1. The most common use of these microarrays is the micro-immunoassay, in which arrays of different capture antibodies are immobilized and subsequently exposed to a biological sample. Analyte proteins bind to the immobilized capture agents and are then detected by fluorescence, luminescence, etc. Various protein-binding agents generated from combinatorial methods may also be used in place of traditional antibodies, such as artificial proteins [32], RNA or DNA aptamers [33], allosteric ribozymes [34], peptides, small molecules, and so on [3•]. The largest number of capture agents to be immobilized on an array was described by Haab *et al.* [20]. They spotted 115 different antibodies onto a slide array, and incubated it with a mixture of fluorescently labeled protein antigens. Sixty percent of the antibodies tested could give a qualitative indication of the presence of their cognate proteins at the highest concentration of antigens tested (1.6 $\mu g\ ml^{-1}$), but only about 23% of the antibodies gave quantitative data at this concentration. The fraction of the antibodies that provided useful signal declined with decreasing antigen concentration, and the presence of large amounts of non-antigen (serum) proteins also decreased the sensitivity of the measurement by increasing the background fluorescence.

An alternative to the detection of labeled analytes is a 'sandwich assay', which utilizes two antibodies that simultaneously bind the same antigen: one antibody is immobilized onto the surface, and the other one is fluorescently labeled or conjugated to an enzyme that can produce a fluorescent, luminescent or colored product when supplied with the appropriate substrate. The disadvantage of this approach is that it requires two specific binding agents for each protein that is to be measured; the advantage is that sample labeling, which can be rather variable between samples [20], is not required; also specificity is higher in sandwich assays because two different reagents must simultaneously recognize the protein to produce signal. Using a sandwich approach, Huang *et al.* [35] have described a simple, low-density filter-based array to measure 25 cytokines in parallel, and achieved detection levels compatible with physiological protein concentrations. Higher-density arrays of antibodies against cytokines and other medically important proteins have also been developed [22••,23,24]. In these cases, the arrays were printed into wells of 96-well microtiter plates, giving microarrays within macroarrays.

Antibody arrays have also been used to capture entire cells. Belov *et al.* [36••] constructed arrays of 60 different antibodies against CD (cluster of differentiation) antigens. Leukocytes from the blood of leukemia patients were exposed to the array, washed, and the pattern of cells binding to the different features was analyzed by light microscopy. Leukocytes express on their plasma membranes different subsets of CD antigens depending on the type of leukemia, so the binding pattern of cells on the array can be used for diagnosis, as well as drug target discovery.

Reverse immunoassays have also been used in microarray formats. In these cases, purified antigens or extracts from tissues or allergenic substances are immobilized on the surface and subsequently probed with antibodies to known proteins [37] or with serum. In the latter case, patients can be diagnosed for various autoimmune diseases [21] or allergies [38,39].

Not only protein abundance, but also activity can be evaluated in microarray formats, provided that the native protein fold is preserved after immobilization [40]. Zhu *et al.* [12] cloned and arrayed into 'nanowells' 119 of the 122 known protein kinases in yeast, and then assayed their specificity by exposing the array to 17 different substrates in the presence of radio-labeled ATP. By following the incorporation of radio-labeled phosphate into the array, they defined the substrate specificity of nearly all the

protein kinases from this organism. In an even larger scale effort [11••], the same group accomplished the monumental task of cloning, expressing, purifying and arraying 80% of the yeast proteome. Each of the 5800 open reading frames was cloned into a yeast expression vector that encodes a fusion protein with glutathione-*S*-transferase and a His-tag. Expression and purification was performed in microtiter plates, and 10–950 femtograms of each protein were arrayed at a density of about 100 features per square mm. A Ni^{2+}-chelating surface was used, thus allowing for oriented attachment of the recombinant proteins. This approach identified 6 of the 12 known calmodulin-binding proteins (the others were either not in the library or were not expressed at detectable levels). A number of lipid-binding proteins were also found. It is clear that the proteome arrays from yeast and other organisms will be exploited for numerous creative applications in the future.

Conclusions and future prospects

Many important developments in the generation and utilization of functional protein microarrays have been reported in the past year. Groups from widely diverse fields, with different expertise, have reported advances in microdevice design, surface chemistry, bioconjugation chemistry and microassay development. Simultaneously, several creative biological applications of this new technology have emerged. Some applications are sufficiently robust that they can be carried out using simple protocols and off-the-shelf reagents, whereas others will require sophisticated chemistry and device design. The more quantitative and delicate systems will benefit from closer collaborations between scientists in these different disciplines, so that the best devices, surfaces, and experimental protocols will be applied to the most important biological and medical problems. To date, multiplexed micro-immunoassays have constituted the most common uses of protein microarrays. Because immunoassays rely the immobilization of fully functional proteins, lessons gained from optimizing these systems will be instructive for the design of more complex micro-analytical devices for the parallel characterization of the enzymatic and binding activities of proteins.

Update

Recent work [43] demonstrates that protein microarray analysis can be combined with laser capture microdissection (LCM), to analyze cell population's tissues containing either normal, pre-malignant, or invasive carcinoma cells. Proteins from the LCM-purified cell types were extracted under denaturing conditions, randomly biotinylated and then incubated on a microarray of 368 different antibodies, most of which were raised against proteins involved in cancer, cell growth or signaling. The arrays were then washed and protein binding was detected based on the biotin tags on the extracted proteins. Using this approach, several proteins were identified as being differentially regulated upon tumor progression. Differences were also found in the stromal tissue surrounding the carcinoma cells. Most of the identified proteins are known to be involved in signal-transduction pathways.

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